Master's Thesis

Development of cancer cell death inducer via interfering MFF-VDAC interaction

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Approved by

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Youngchan Chae

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This certifies that the thesis/dissertation of Nuri Lim is approved.

12/11/2020

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Eunhee Kim

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Abstract

Mitochondria regulate energy metabolism, biosynthesis, cell death, redox homeostasis and signaling pathway and dysregulated mitochondria abundantly observed in cancer. Therefore, researchers tried to targeting mitochondria for killing cancer. The mitochondrial fission factor (MFF) and voltage-dependent anion channel (VDAC) form a complex which of interruption activates mitochondrial outer membrane permeability (MOMP) and induces cell death. MFF peptide, synthetic MFF sequence peptide that competitively inhibit MFF and VDAC interaction, treatment leads to mitochondrial cell death in cancer but not in normal. However, due to poor stability of peptide in vivo, it is needed to develop new substance that inducing cell death via interfering of MFF-VDAC interaction like MFF peptide. We developed chemical screening system to seek chemical interfering MFF-VDAC interaction using nanobit protein-protein interaction assay system which is structural complementation reporter system. The chemical identified from this assay system could be a new anti-cancer drug that is targeting mitochondria to induce cancer cell death.

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Abbreviations

MOM : Mitochondrial Outer Membrane

MOMP : Mitochondrial Outer Membrane Permeabilization

PCD : Programmed Cell Death

MFF : Mitochondrial Fission Factor

VDAC : Voltage-Dependent Associated Channel

TCA cycle : Tricarboxylic Acid Cycle

OXPHOS : Oxidative Phosphorylation

FAO : Fatty Acid Oxidation

PPI : Protein-Protein Interaction

IDH1/2 : Isocitrate Dehydrogenase 1/2

D-2HG : D-2 Hydroxyglutarate

AML : Acute Myeloid Leukemia

SHMT2 : Serine Hydroxymethyltransferase 2

TMRE : Tetramethylrhodamine, Ethyl Ester, Perchlorate

TAP-MS : Tandem Affinity Purification-Mass Spectroscopy

I. Background

1.1 Mitochondria and cancer

In the past, there is a misunderstanding that mitochondria activity would not be important in cancer because of 'Warburg effect' that cancer use glycolysis for energy production despite presence of oxygen for mitochondrial respiration. However, continuous studies revealed that mitochondrial respiration still possess high proportion of energy production in cancer which display Warburg metabolism and even some cancer have high dependence of mitochondrial respiration.

Typically, Tumor cells form tumor mass through uncontrollable and undiscriminating cell proliferation. As tumor mass become bigger and bigger, they encounter various cellular stress such as hypoxia, inflammation, nutrient deprivation, and oxidative stress. Also, cancer meets these stresses during progression. Thus, for cancer to develop and progress to next stage, they must overcome harsh environment arose from cellular stresses. In this situation, mitochondria contribute to enduring cellular stresses and support cancer to initiate and progress (Figure 1.1). For example, cancer produce energy from mitochondria when cancer are in nutrient-deprived condition.

The major role of mitochondria is producing energy through oxidation phosphorylation (OXPHOS) pathway connected with tricarboxylic acid cycle (TCA cycle) and Fatty acid oxidation (FAO).[1] Cancer prefer to use glycolysis for energy production to support rapid proliferation, therefore, cancer consume lots of glucose.[2] However, even though cancer conjugate glycolysis, it's not means cancer do not use mitochondria because of efficiency of energy production and flexible alteration of mitochondrial metabolism. Furthermore, when tumor mass encounter nutrient-deprived condition by excessive growth, tumor cells can use other carbon sources such as lactate, acetate, glycine, and serine for mitochondrial energy production (Figure 1.2).[3,4,5] And recent researches show that some cancer type or metastatic cancer have high dependence of mitochondrial energy production.[6,7] It means that mitochondria is essential organelle to cancer cell to survive in harsh environment and progress to next stage.

1.2 Mitochondrial Outer Membrane Permeabilization (MOMP) : the door of programmed cell death

In general, normal cells undergo programmed cell death when cells get severe damage from cellular stresses. The death signaling are activated in response to cellular stresses and initiate apoptosis

through mitochondrial outer membrane permeabilization (MOMP). Once MOMP is triggered in cells, there is nothing to return apoptosis back. Thus, MOMP is known as the point of no return for apoptosis. At this point, mitochondria play central role as mediator of MOMP. MOMP occurred by activating pro-apoptotic Bcl2 family proteins : Bax and Bak. The death signaling activates Bax to make pore in mitochondrial outer membrane via homo-oligomerization or hetero-oligomerization with Bak and VDAC (Figure 1.4). This pore releases mitochondrial intraspace proteins such as SMAC and cytochrome c. Then, they activate caspases that is executer of cell degradation (Figure 1.3).

1.3 A Cancer Hallmark : Avoiding cell death

Cancer could avoid the cell death, which is one of cancer hallmarks, blocking MOMP through various ways. As mentioned above, normal cells activate MOMP in response to intrinsic lethal stimuli. However, in cancer cell, despite of intrinsic lethal stimuli, Bax and Bak failed to forming pores in several ways. Then, cancer avoid cell death. A way of avoiding cell death is upregulating anti-apoptotic proteins and inhibitor of apoptosis proteins. They inhibit the activation of Bax and Bak blocking pore construction. Also, downregulation and silence mutation of pro-apoptotic proteins prevent Bax and Bak to make pores (Figure 1.5). Thus, failure to forming pores prevent MOMP then, finally cancer cell could avoid cell death in presence of intrinsic lethal stimuli. Furthermore, cancer cells appear drug resistance by preventing MOMP.

1.4 Targeting mitochondria for treatment of cancer

Recent researches revealed that mitochondria is dysregulated by overexpression of oncogenes and loss function of tumor suppressor genes in cancer. Dysregulated mitochondria support initiation of tumorigenesis and tumor cell survival encountered harsh environment such as hypoxia, nutrient-deprived condition, and metabolic stress.[32] Some cancers are highly dependent on mitochondrial metabolism and inhibition of mitochondrial activity promotes repression of cell proliferation and cell death.[33] As conquering dysregulated mitochondria mechanism and vulnerabilities, targeting mitochondria could be a new therapeutic strategy.

1.5 Figures

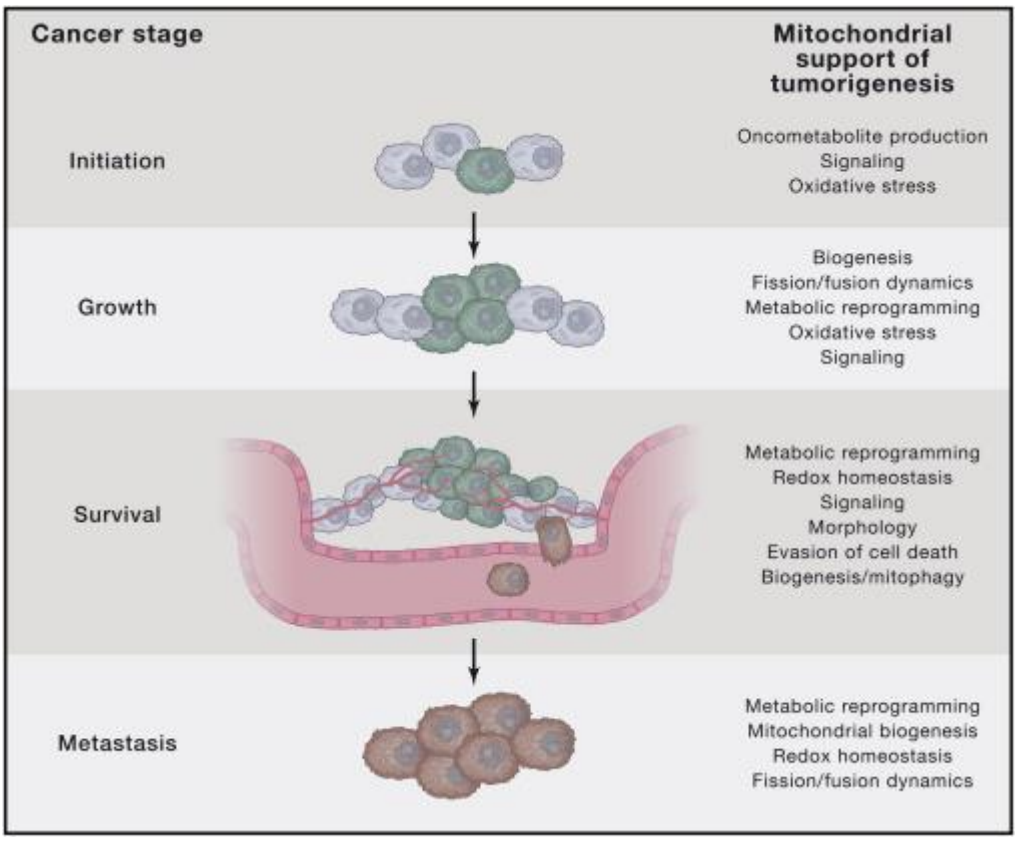

Figure 1.1 Mitochondria support cancer at multiple stages

(2016). Mitochondria and Cancer. *Cell*, *166*(3), 555–566.

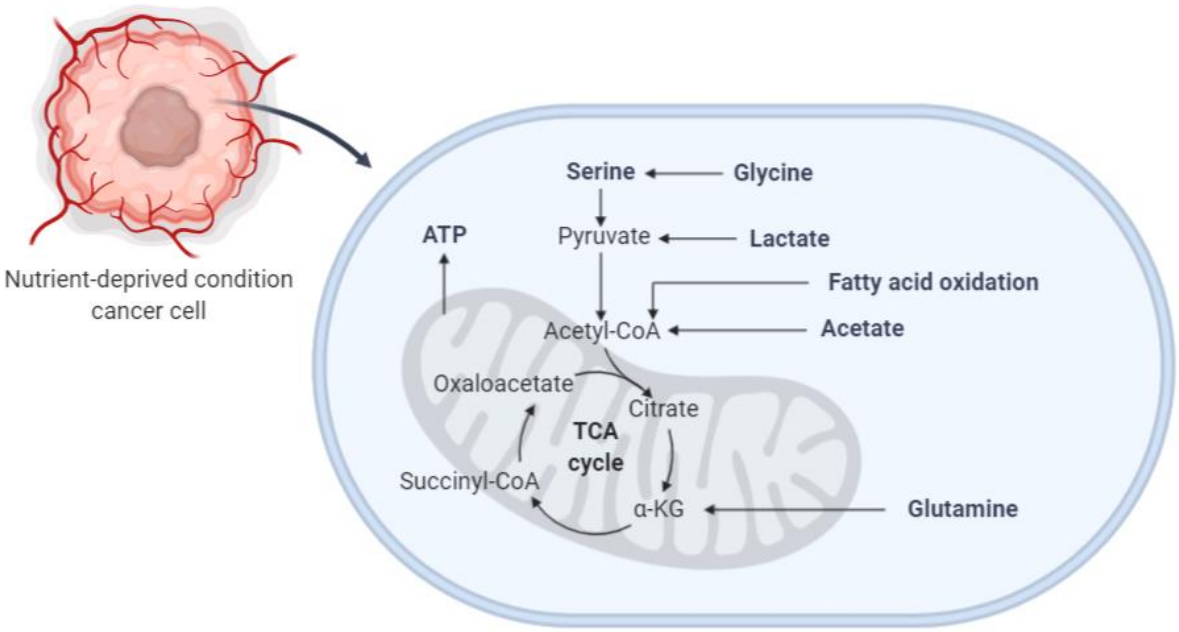

Figure 1.2 Cancer cell mitochondrial metabolism in nutrient-deprived conditions

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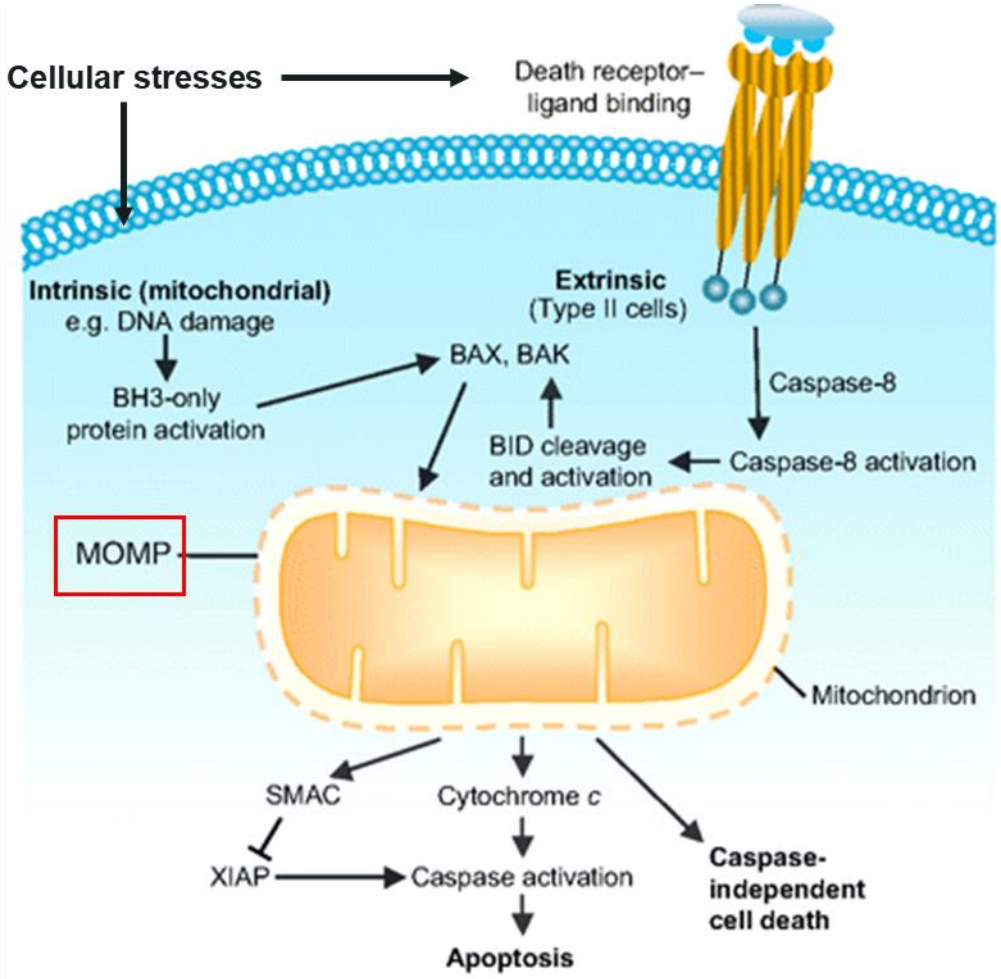

Figure 1.3 Mitochondrial outer membrane permeabilization induces cell death

(2012). Mitochondria and cell signalling. *Journal of Cell Science*, *125*(4), 807–815.

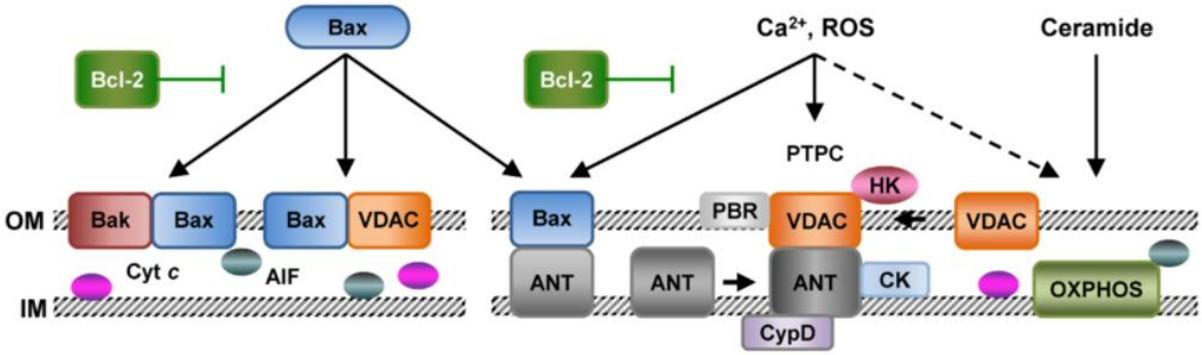

Figure 1.4 Bax makes pore in mitochondrial outer membrane via homo-oligomerization or hetero-oligomerization with Bak and VDAC

(2008). Viral Control of Mitochondrial Apoptosis. *PLOS Pathogens*, *4*(5), e1000018.

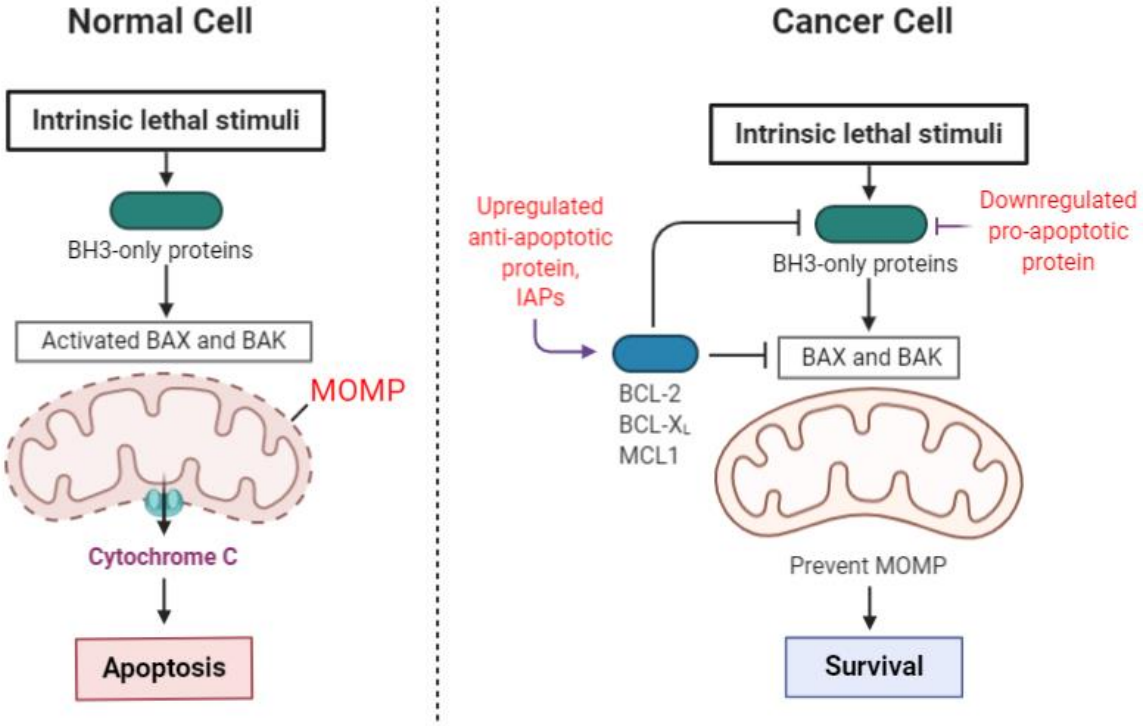

Figure 1.5 Cancer avoid cell death through several ways in presence of intrinsic lethal stimuli

(2019). Contribution of Mitochondrial Ion Channels to Chemo-Resistance in Cancer Cells. *Cancers*, *11*(6), 761

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II. Development cancer cell death inducer via interfering MFF-VDAC interaction

2.1 Introduction

Mitochondria regulate several kinds of programmed cell death (PCD) such as apoptosis and necroptosis.[39,40] And PCD is essential natural mechanism and one of defense mechanism in response to severe DNA damage, pathogen infection and uncontrolled cell proliferation. Thereby, tissues and cells maintain cell homeostasis and healthy condition preventing excessive cell proliferation and a series of infection. Since PCD is determines survival or death of cells, it tightly regulated by cell signaling pathway that mitochondria is centered. Among PCD process, mitochondrial outer membrane permeabilization (MOMP) is most important step known as the point of no return because nothing can take apoptotic signaling back once stimulated.[41] However, cancer have ability to avoid apoptosis through dysregulated mitochondria. Therefore, researchers tried to targeting mitochondria for cancer therapy inducing PCD.

Our laboratory previously studied trying to find effective anti-cancer drug targeting mitochondria. The central topic was mitochondrial fission factor (MFF) known as regulator of mitochondrial fission with Drp1 and overexpressed in prostate cancer. The proteomics result for MFF-associated proteins shows MFF interacts with VDAC that forms MOMP regulating complex.[42] Indeed, regulation of MFF by siRNA shows reduction of mitochondrial potential. This result suggests the unknown function of MFF in cancer.

The Nanobit PPI (protein-protein interaction) assay used to develop cancer cell death inducer screening system to search chemical act like MFF peptide. Nanobit PPI assay is structural complementation reporter system composed of two subunits; one is Large bit (18kDa) and the other is small complimentary peptide (11 amino acid peptide). Each subunit can tag to interest of protein and express in cell line. When the two proteins interact in cell, the nanobit subunits also interact together generating luminescent signal in the presence of substrate.[43] Furthermore, two subunits interaction is reversible, enabling detection of luminescent signal in real time and even detectable in live cell.

Here, we found MFF and VDAC interact with each other and interfering with interaction induces cancer cell death. In turn, we developed chemical screening system to find chemical like MFF peptide which is suggested to anti-cancer drug that is new targeting in mitochondria.

2.2 Materials and Methods

Antibodies

The following antibodies to Mitochondrial fission factor (MFF, Protein Tech, 17090-1-AP), Voltage-dependent anion channel (VDAC, Abcam, ab15895), β-actin (GeneTex, GTX629630), Flag (Cell signaling, 14793) were used.

Cell culture

DU145 and PC3 cells cultured in Roswell Park Memorial Institute (RPMI 1640), with L-Glutamine and 25mM HEPES, 1L (Lonza, 12-702f) containing 10% Fetal Bovine Serum (Gibco, 16000044) and 1% antibiotics (100ml antibiotic-antimycotic, 100x, Gibco, 15240062) at 37 °C under a humidified 5% CO_2 atmosphere. Ct26, B16F10 and HEK293 cells cultured in Dulbecco's Modified Eagle's Medium (DMEM) and other conditions identical with above. All cells were purchased from the American Type Culture (ATCC).

Cell growth and Cell death measurement

MFF peptide treated cells were detached using trypsin-EDTA (0.25%, Gibco, 25200056) and suspended cell were blended with Trypan blue stain (0.4%, Gibco, 15250-061) with equal volume. 10μl of blended cells were filled in Countess™ cell counting chamber slides (Thermo Fischer Scientific, C10228) and counted by Countess™ FL automated cell counter. Counted variable cell number divided into control cell number and calculated in percentage.

Transfection

For gene knockdown transfection, siRNA mixed with RNAimax (Thermo Fischer Sciencific, 13778030) in the Opti-MEM (Gibco, 31985070) and incubates for 20 min. Mixture added to culturing media. For overexpression transfection, plasmid and jetPEI (polyplus, 101-10N) mixed by mass ratio 1:2 with NaCl solution. After incubating for 15 min, mixture added to cultured media without antibiotics. After 4h, media change to fresh.

Western blot analysis

Protein lysate were prepared in radioimmunoprecipitation (RIPA) buffer (25 mM Tris-HCl/pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, 1% SDS, Biomax) containing a cocktail of proteinase inhibitors and phosphatase inhibitors (100x, Thermo Fischer Scientific, 1861281). And cells are harvested. The concentration of total proteins was measured with BCA reagent (Thermo Fischer Scientific, 23235). Loaded proteins were separated with 8~13% SDS-PAGE and transferred to PVDF membranes (Millipore Sigma, 88518). 5% (w/v) skim-milk was used for blocking and primary antibodies, which has refereed, were incubated overnight at 4°C. After washing with Tris-buffered saline containing Tween 20 (TBS-T) for 3 times every each 10 min, the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies for 1 hour and visualized by ECL and ECL prime reagents (GE Healthcare, RPN2232).

Immunoprecipitation

IP buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA) containing 1% CHAPS, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) and Phosphatase Inhibitor Cocktail PhosSTOP (Roche) were prepared. And cells were harvested and lysed. Then, lysates were sonicated and centrifuged at 15,000 x g for 30 min. And cell lysates were incubated with anti-Flag-conjugated beads (Sigma-Aldrich). The precipitates were washed five times with PBS, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and same process proceeded with western blotting.

Plasmid preparation

The pBiT vectors (pBiT1.1-N[TK/LgBiT], pBiT1.1-C[TK/LgBiT], pBiT2.1-N[TK/SmBiT], pBiT2.1-C[TK/SmBiT]) were purchased from Promega (NanoBiT® PPI MCS starter System Vectors, N2014). MFF and VDAC genes cloned into each pBiT vectors using BiT-MFF primers (5'-CCGCTCGAGCGGTATGGCAGAAATTAGTCGAATTCAG-3' and 5'-CTAGCTAGCCTAGCGGCGAAACCAGAGCCA-3'), MFF-BiT primers (5'-CTAGCTAGCATGGCAGAAATTAGTCGAATTCAG-3' and 5'-CCGCTCGAGCCGCGGCGAAACCAGAGCCAGCT-3'), BiT-VDAC primers (5'-CCGCTCGAGCGGTATGGCTGTGCCACCCACGTATGC-3' and 5'-CTAGCTAGCTTATGCTTGAAATTCCAGTCCTAG-3'), VDAC-BiT primers (5'-CTAGCTAGCATGGCTGTGCCACCCACGTATGC-3' and 5'-CGGCTCGAGCCTGCTTGAAATTCCAGTCCTAGACC-3'). Then, for high plasmid expression level in cells, nanobit tagged genes were cloned into pcDNA3.1(+) vectors.

Nanobit PPI assay

Cells plated on white 96 well plate for measurement of luminescent signal. Preparing the desired amount of reconstituted Nano-Glo® Live Cell Reagent by combining 1 volume of Nano-Glo® Live Cell Substrate with 19 volumes of Nano-Glo® LCS Dilution Buffer (a 20-foled dilution), creating a 5X stock to mix with cell culture medium. Then measuring baseline luminescence and right after treat peptides and with time intervals.

TMRE assay

Cells are plated on black 96 well cell plate. The final concentration 100 nM TMRE (Thermofisher, T669) added to cultured medium with cell for staining. After 20 min, washing 3 times with PBS. 10 min treatment of peptides and measuring fluorescence signal or take image by fluorescence microscopy.

2.3 Results

MFF and VDAC interact with each other and MFF peptide treatment interferes with MFF-VDAC interaction

In previous study of our laboratory, the proteomic result suggests MFF and VDAC may interact with each other forming MOMP regulating complex at mitochondrial outer membrane. First, we confirmed MFF and VDAC interaction by co-immunoprecipitation. We expressed flag tagged MFF or VDAC, respectively with normal partner protein, and pulled down by flag antibody. As a result, flag-MFF interacted with VDAC and flag-VDAC interacted with MFF demonstrating MFF and VDAC interact with each other (Figure 2.1A and 2.1B). Furthermore, when flag-VDAC and GFP-MFF co-expressed and pulled down by flag antibody, MFF was detected by GFP antibody (Figure 2.1C). It provides high reliability clue that MFF-VDAC interact with each other. In previous study, we found a part of MFF sequence, the MFF peptide that reduced mitochondrial membrane potential similar with siMFF treatment. Then, we investigated whether MFF peptide treatment affect to MFF-VDAC interaction. We treated scr peptide and MFF peptide in flag-VDAC expressed cells and observed MFF expression in co-immunoprecipitation with flag antibody. Then, we observed that pulled down MFF with flag antibody was decreased in MFF peptide treatment contrast to scr peptide treatment (Figure 2.1D). Therefore, MFF peptide treatment interferes with MFF-VDAC interaction.

MFF peptide treatment induces cell death through activating MOMP

Then, to investigate whether MFF-VDAC interaction affects to cell viability, we observed cell growth and cell death with MFF peptide dese-dependent treatment. Cell growth were measured by percentage of lived cell number contrast to before treated, and cell death were measured by percentage of dead cell number contrast to total cell number. The rate of cell growth decreased by treatment of MFF peptide dose-dependently (Figure 2.2A). And cell death increased with same experimental condition of cell growth (Figure 2.2B). Because programmed cell death is known to be initiated by MOMP, we measured mitochondrial membrane potential by staining TMRE (Figure 2.2C - 2.2E). MFF peptide treatment decreased mitochondrial membrane potential coupled with MOMP suggesting MFF peptide treatment promotes MOMP-induced cell death.

LgBiT-MFF and SmBiT-VDAC are proper combination to express highest luminescent activity

Since MFF peptide has several disadvantages such as high production cost, in vivo instability and low membrane permeability, it depreciates the value as anti-cancer drug. Therefore, we need to search substance that interfering MFF-VDAC complex like MFF peptide and simultaneously covers disadvantages of MFF peptide. To search wanted substance, we developed chemical screening system using Nanobit PPI assay which system is detecting chemicals that interfering MFF-VDAC interaction like MFF peptide in live cell (Figure 2.5A). To construct a chemical screening system with Nanobit PPI assay, the two subunits must interact with each other properly. However, since we do not know structure of MFF and VDAC, and how they interact with, we have to consider the possibilities of all combinations of subunits. Thus, we produced nanobit subunit tagged protein plasmid and tuned the expression level of nanobit tagged proteins as similar as possible (Figure 2.5B) because we have to find best combination which generate highest luminescent activity in same numbers of combination. Combinations that are composed with VDAC-LgBit did not shown in data because of poor expression level.

To discriminate which combination is the most suitable combination, the possibilities of all combinations were expressed in cells and the luminescent activity was measured. As a result, the combination of LgBiT-MFF and SmBiT-VDAC showed the highest activity over time than other combinations. Therefore, we confirmed that LgBiT-MFF and SmBiT-VDAC are the most optimal combination for chemical screening system (Figure 2.5C).

MFF peptide efficiently decreases luminescent activity interfere with MFF-VDAC interaction

Because we determined the most optimal combination for screening, we need to confirm whether luminescent activity is decreased by MFF peptide treatment. Therefore, we measured luminescent activity at LgBiT-MFF and SmBiT-VDAC co-expressed in cell with treatment of MFF peptide (Figure 2.6A). Before treatment of peptide, the luminescent activities of all samples are similar level (Figure 2.6B). However, luminescent activities are decreased by dose-dependently in MFF peptide treated cells after 120 minutes (Figure 2.6C). Furthermore, we confirmed MFF peptide function in essay used cell line. Decreased TMRE staining in MFF peptide treated cells suggest interfering of MFF-VDAC interaction induces MOMP (Figure 2.7A, B and C).

2.4 Discussion

Mitochondria have important role in cancer from initiation to metastasis. Various role of mitochondria such as metabolic reprogramming, redox homeostasis, evasion of cell death, biogenesis and mitochondrial dynamics supports cancer cells to survive in any circumstances.[32] Therefore, targeting mitochondrial is one of strategy for cancer therapy. There are some therapeutic targets in mitochondria. Mutated IDH1/2 (isocitrate dehydrogenase; 1 in cytosol and 2 in mitochondria) produce D-2 hydroxyglutarate (D-2HG) known as a oncometabolite and mostly detected in acute myeloid leukemia (AML), bile duct cancer, bone sarcoma and glioma. They contribute to tumor initiation and progression through blocking cell differentiation and could be an anti-tumor target.[34,35] As mentioned above, one carbon metabolism serves mitochondrial biomass synthesis. Since SHMT2 (serine hydroxymethyltransferase 2) is a crucial enzyme in synthesis of serine and glycine, inhibition of SHMT2 could block biomass synthesis and some research revealed that SHMT2 inhibition reduced cancer cell growth.[36,37] Several anti-cancer drugs targeting mitochondrial OXPHOS pathway are widely used already.[38]

There are several assays for investigating PPI such as tandem affinity purification-mass spectroscopy (TAP-MS), affinity chromatography, co-immunoprecipitation and protein microarrays. Nanobit PPI assay has some advantages contrast to other PPI assay. First, nanobit subunits have small affinity with each other providing high accuracy of protein-protein interaction. And due to nanobit subunits interact reversibly, it allows detecting protein-protein interaction in real time and even detectable in live cell condition.

In case of our study, we figured out best combination of nanobit subunits in MFF and VDAC interaction. And we confirmed MFF peptide greatly decreases luminescent activity by interfering MFF-VDAC interaction. As mentioned above, we need to identify MFF peptide like substance using chemical screening system we developed. There are some chemical libraries for testing our system. Next, we could figure out several chemicals that decreasing luminescent signaling in our system. That means chemicals effectively interfere with MFF-VDAC interaction. Then, we have to pick out optimal chemical through several experiments that proceeded with MFF peptide such as cell death rate and TMRE staining. The figured-out chemical could be a potential anti-cancer drug that effectively kill the cancer cells further promoting effects of anti-cancer immune system.

2.5 Figures

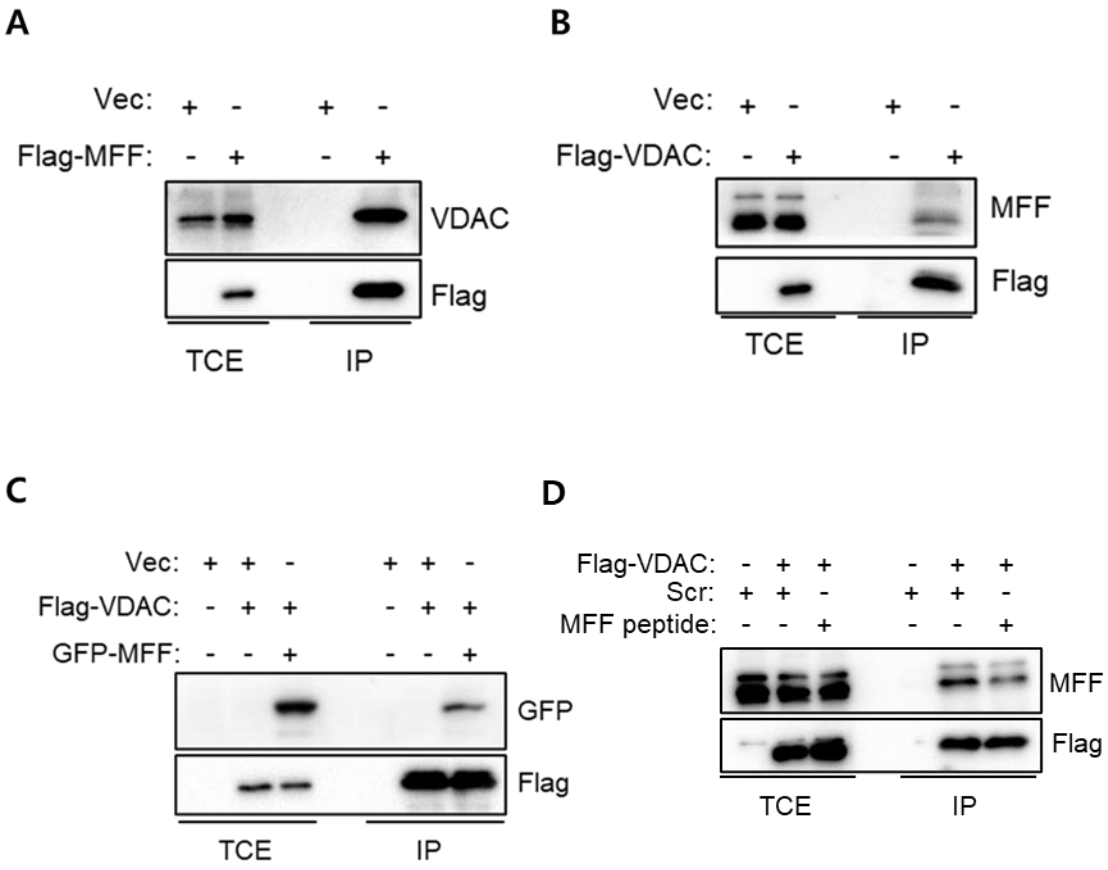


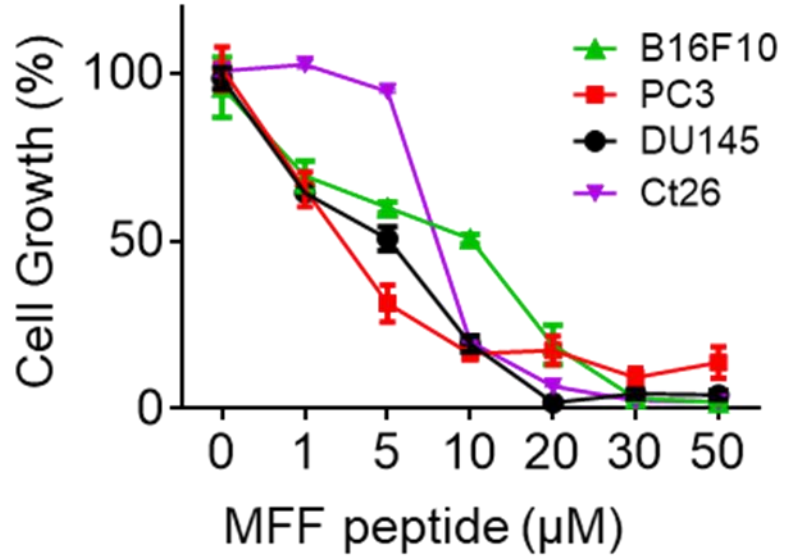
Figure 2.1 MFF and VDAC interact with each other.

(A-B) PC3 cells transfected with vector, Flag-MFF (A) or Flag-VDAC (B) were immunoprecipitated (IP) with an antibody to Flag and analyzed by western blotting.

(C) PC3 cells transfected with vector, Flag-VDAC, GFP-MFF were immunoprecipitated with an antibody to Flag and analyzed by western blotting.

(D) PC3 cells transfected with vector and Flag-VDAC and treated scr peptide or MFF peptide were immunoprecipitated with an antibody to Flag and analyzed by western blotting.

A



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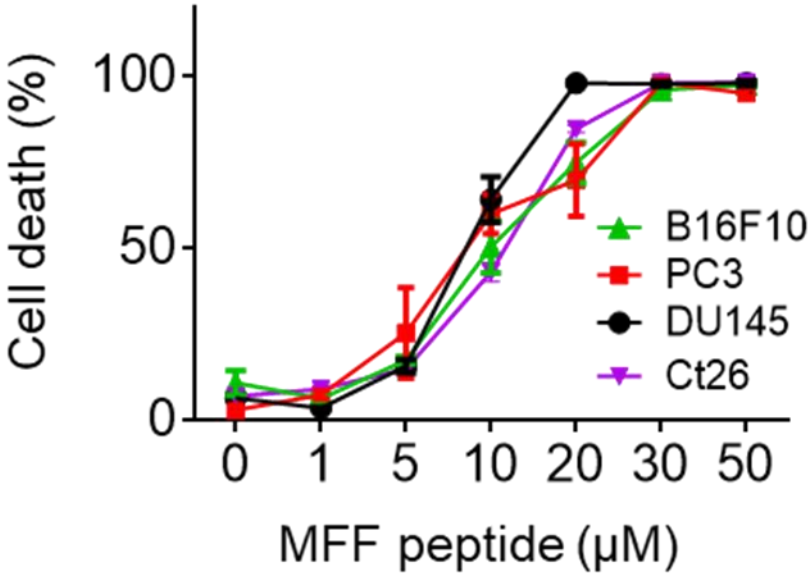

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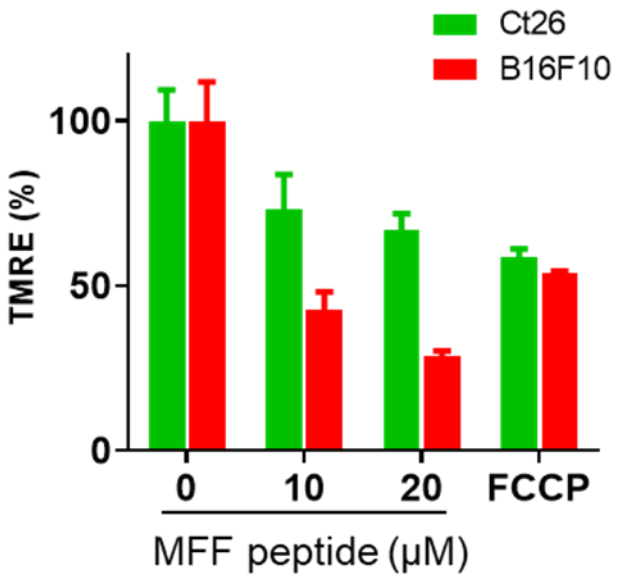

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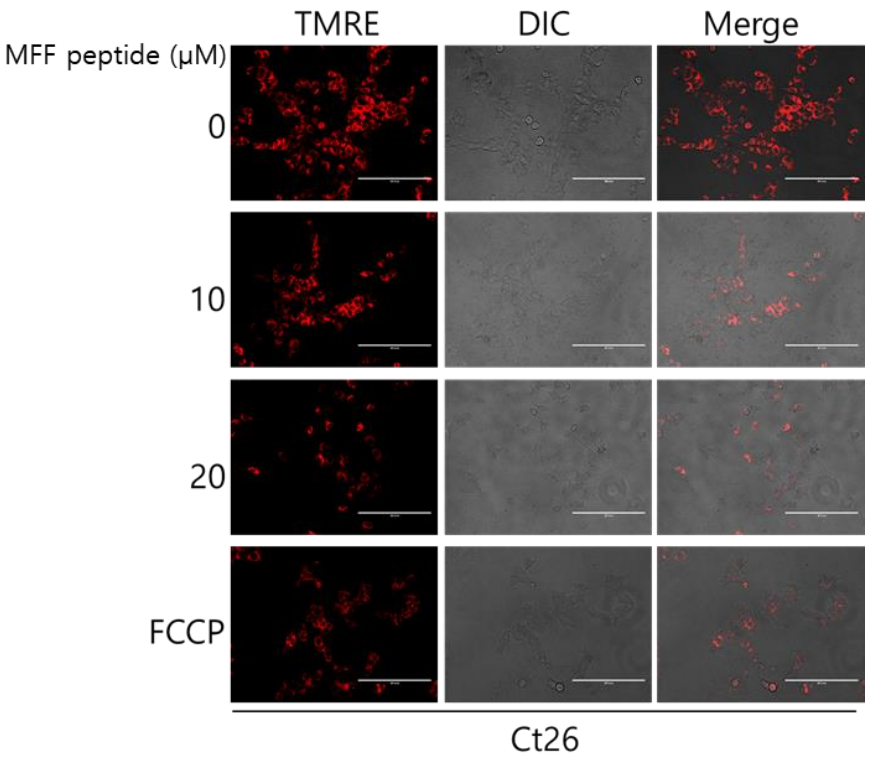

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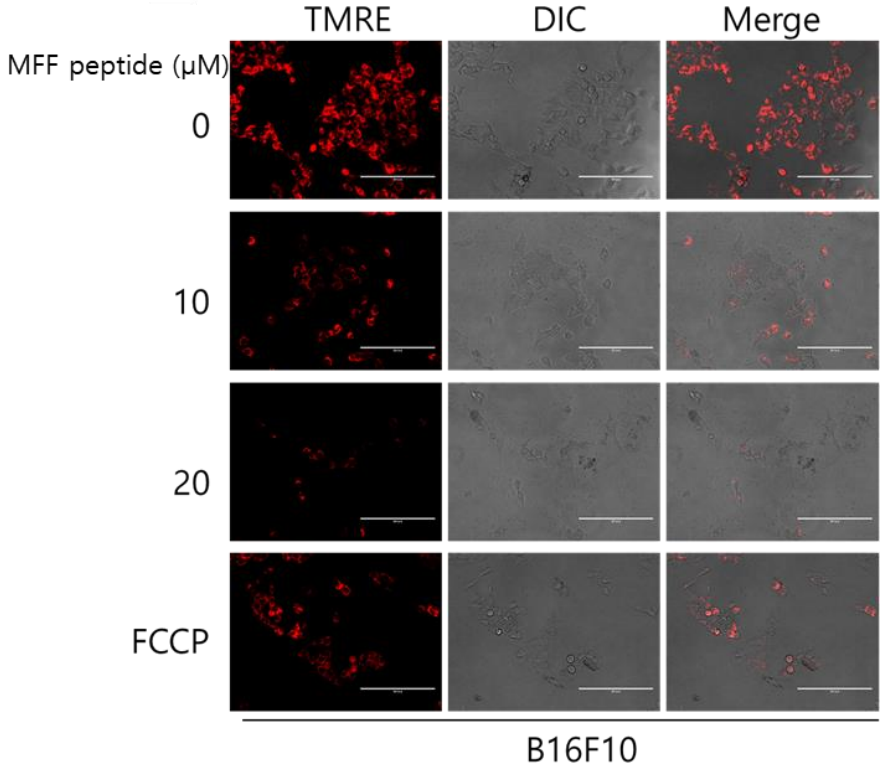


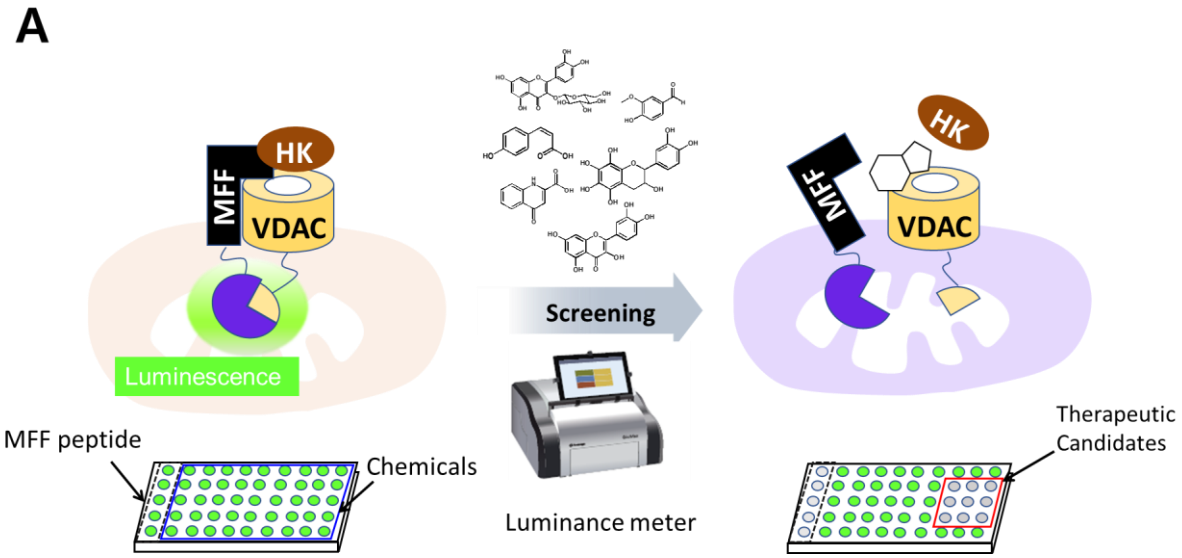
Figure 2.2 MFF peptide treatment decreases cell growth and increases cell death through MOMP

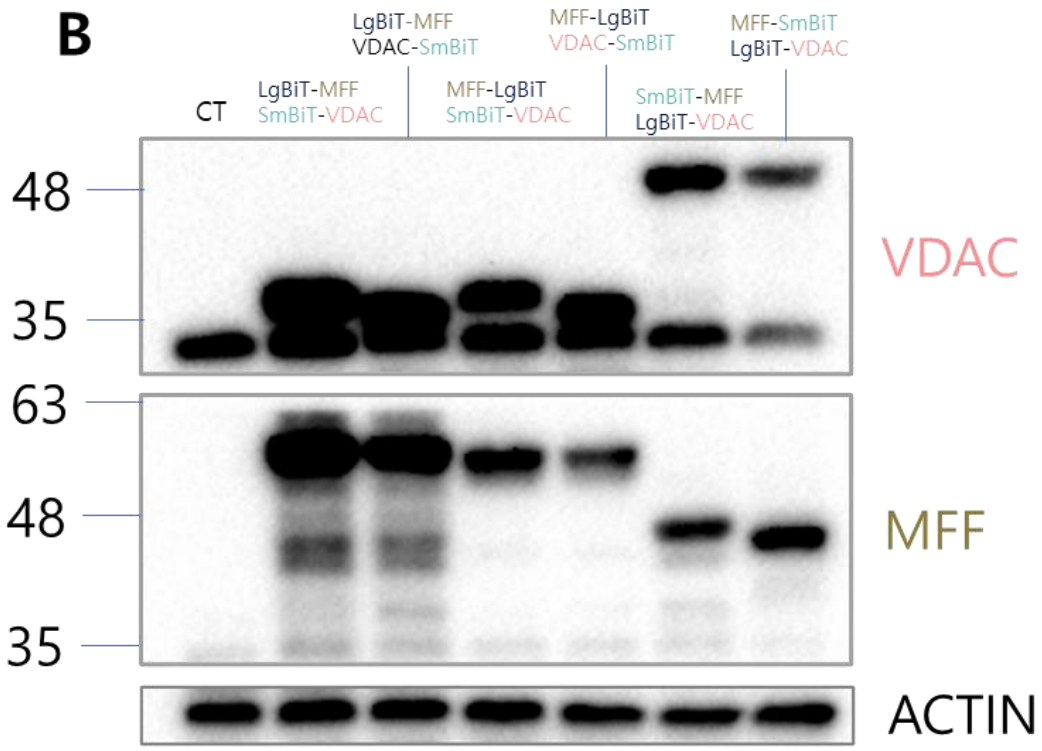
(A) Cell growth were measured by percentage of lived cell number contrast to before treated MFF peptide.

(B) Cell death was measured by percentage of dead cell number contrast to total cell number at each MFF peptide dose.

(C) MFF peptide treatment in Ct26 and B16F10 cell lines analyzed for mitochondrial membrane potential analyzed by TMRE staining. FCCP was used as a control.

(D-E) Fluorescence microscopy images of Ct26 and B16F10 treated MFF peptide and FCCP showing mitochondrial membrane potential by TMRE staining.





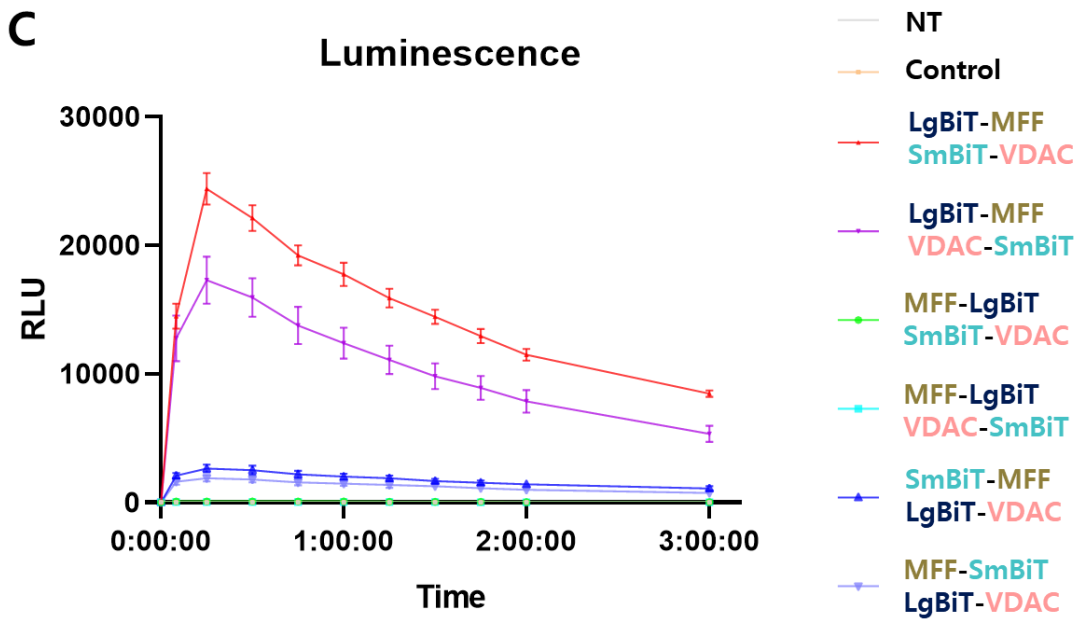
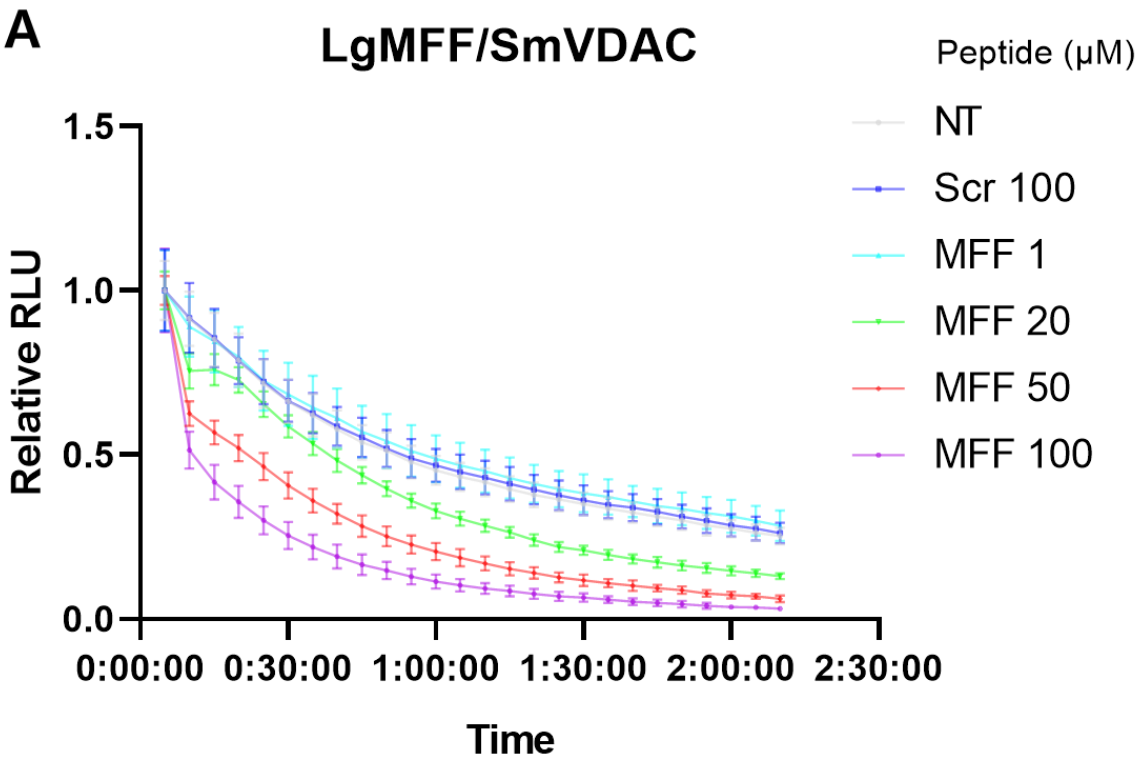


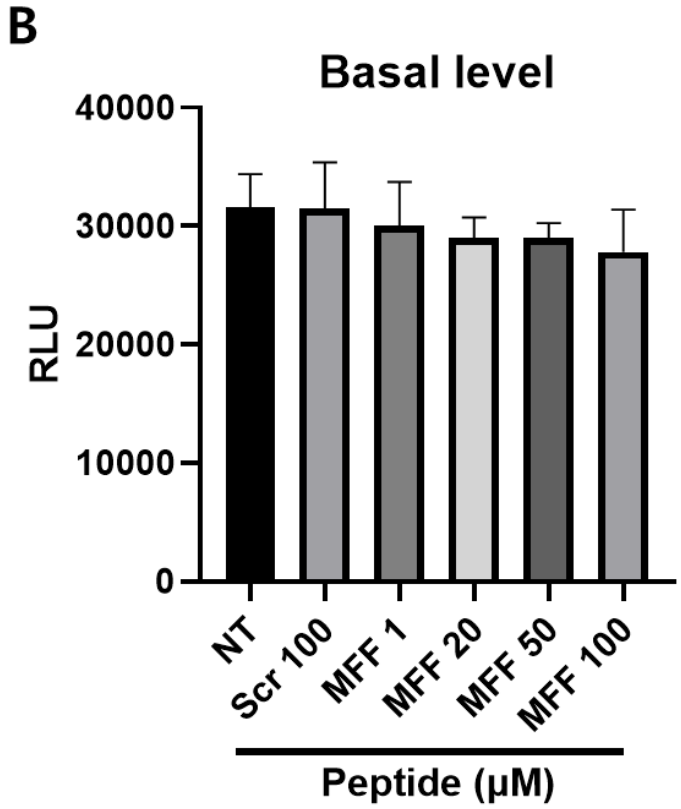
Figure 2.3 LgBiT-MFF and SmBiT-VDAC are proper combination to express highest luminescent activity

(A) The scheme of chemical screening system using nanobit PPI assay.

(B) All possibilities of nanobit combination transfected to HEK293 cell line

(C) All possibilities of nanobit combination measured luminescent activity in HEK293 cell line.





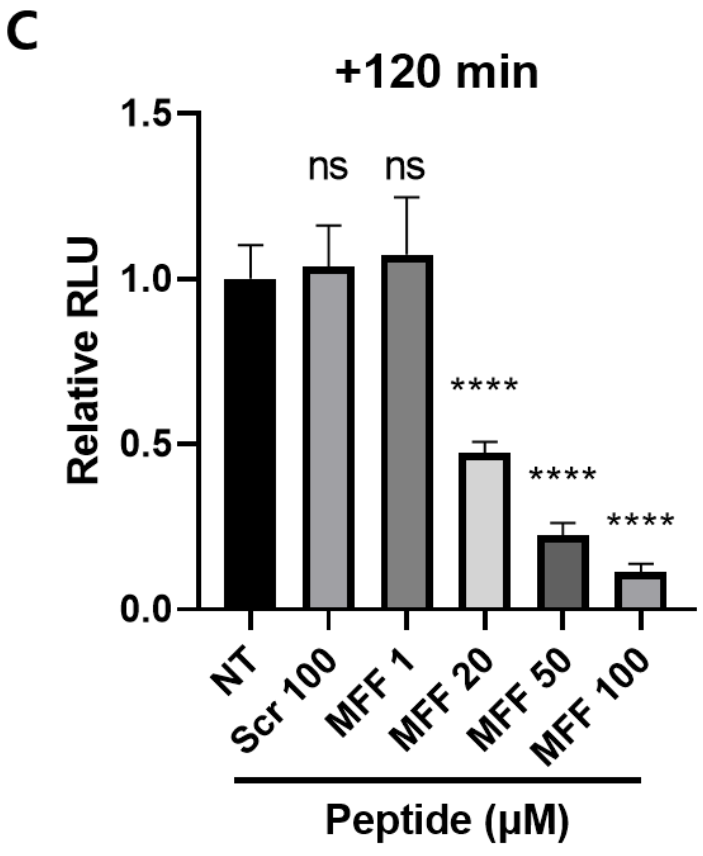
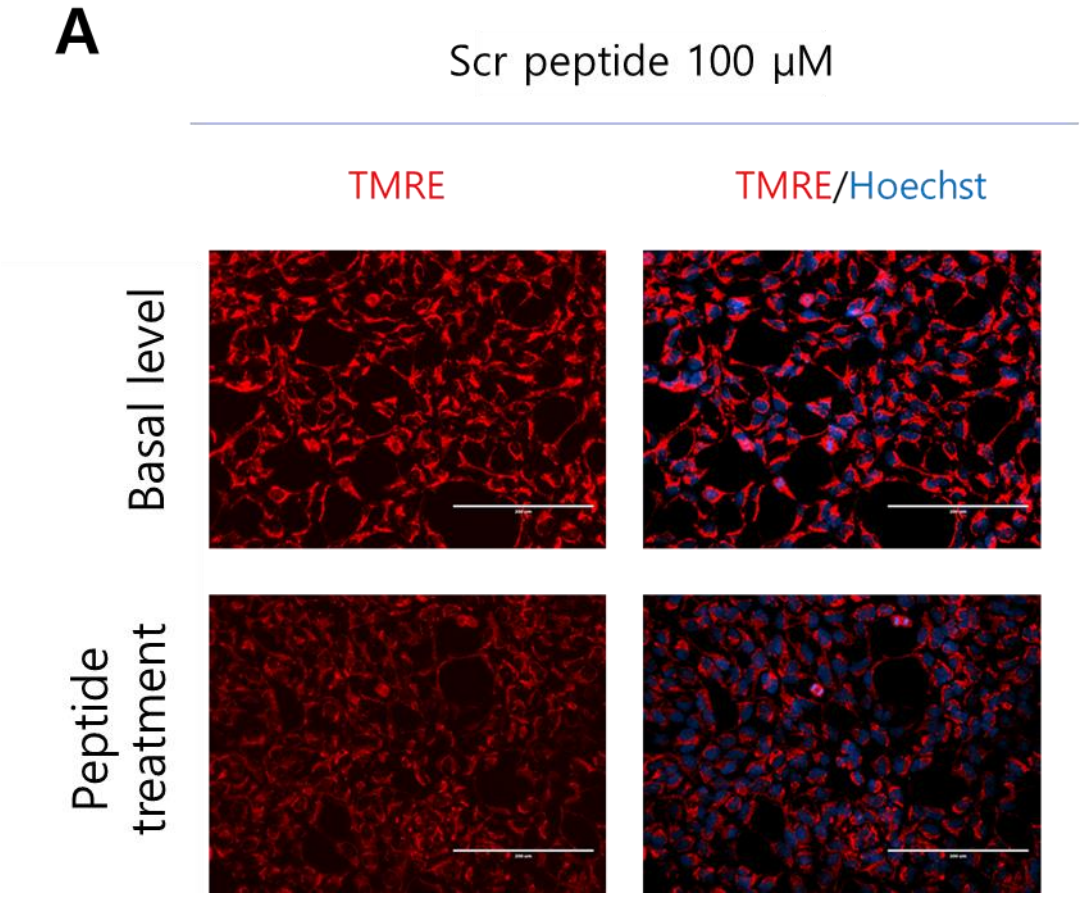


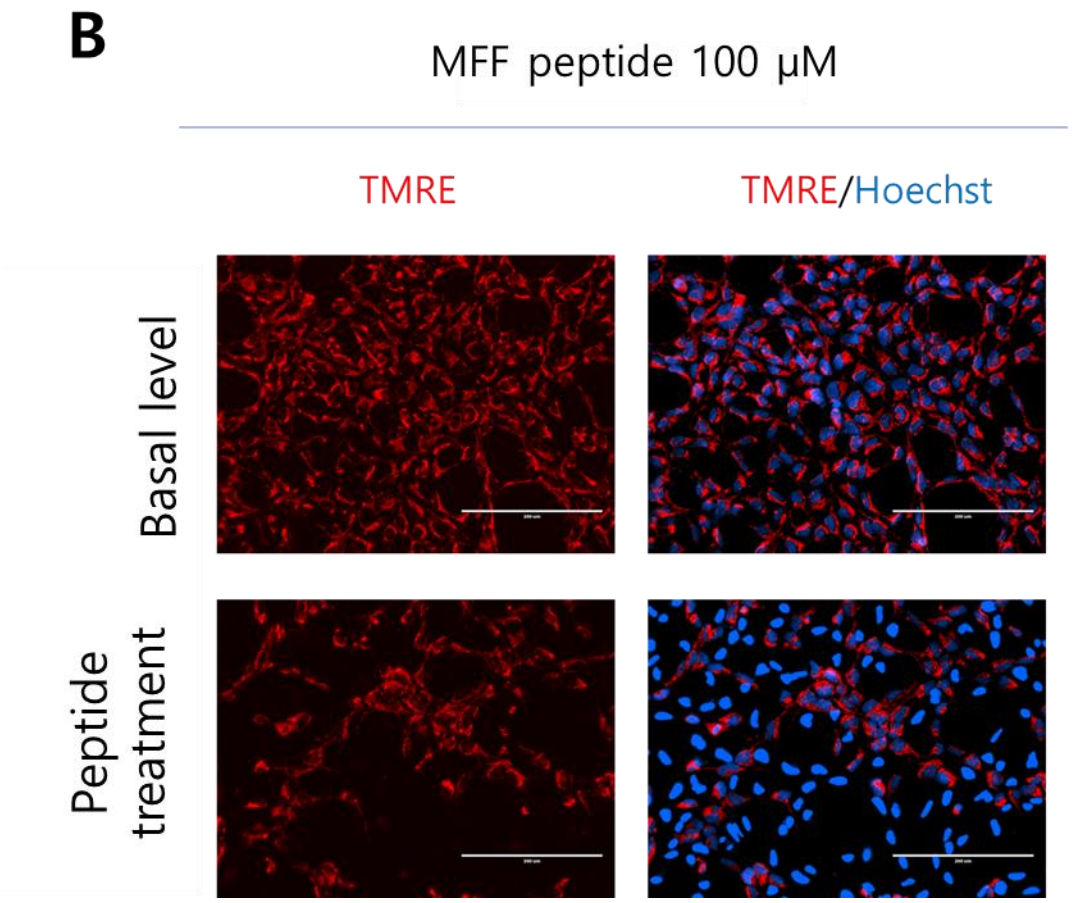
Figure 2.4 MFF peptide efficiently decreases luminescent activity interfere with MFF-VDAC interaction

(A) Relative luminescent activity measured by time lapse in LgBiT-MFF and SmBiT-VDAC transfected HEK293 cell line

(B) Luminescent activity measured when immediately nanobit substrate is added.

(C) Relative luminescent activity measured in peptide treated cells after 120 minutes.





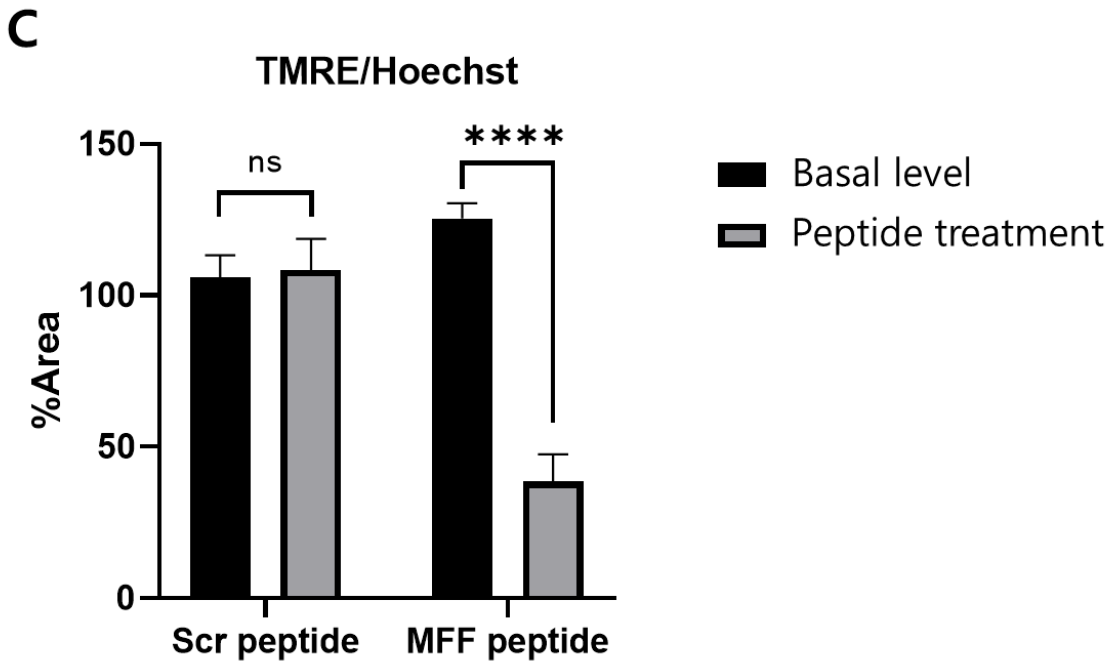


Figure 2.5 MFF peptide treatment decreased mitochondrial activity in HEK293 cell line

(A-B) Fluorescence microscopy images of Ct26 and B16F10 treated MFF peptide showing TMRE staining. Cell nucleus were stained with Hoechst 33342.

(C) The differences of %area (TMRE/Hoechst) between Scr peptide and MFF peptide.

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